J. Physiol. (1963), **165**, *pp.* 421–436
With 4 text-figures
Printed in Great Britain

DETERMINATION OF IONTOPHORETIC RELEASE OF ACETYLCHOLINE FROM MICROPIPETTES

By K. KRNJEVIĆ, J. F. MITCHELL* AND J. C. SZERB†
From the A.R.C. Institute of Animal Physiology, Babraham, Cambridge

(*Received* 25 *April* 1962)

The application of ionized substances to a nerve or muscle cell by passing a current through a micropipette was first described by Nastuk (1953). This technique is particularly convenient because it can be accurately localized both with respect to time and place. It is also quantitative, since the amount of substance applied should be proportional to the total charge passed through the pipette.

Although the method has been adopted widely, there seems to have been no direct check on how much substance is actually released. We have therefore examined some properties of single and multibarrelled micropipettes which contained strong solutions of acetylcholine (ACh). Measurements were done of the amounts of ACh released *in vitro* under conditions approximating as nearly as possible to those in physiological experiments, within the limitations imposed by the sensitivity and accuracy of the available methods of biological assay. An attempt was made to see how far the results could be fitted into a theoretical framework.

METHODS

Single micropipettes. Four millimetre Pyrex tubing, with a 2 mm bore, was drawn out by a device similar to that described by Winsbury (1954). The pipettes were filled with glass-distilled water by boiling under a reduced pressure at 70° C for about 20 min. After cooling, the bulk of the water was sucked out by means of a fine polythene tube and replaced with 3·0 M AChCl (Roche Products). The pipettes were then stored in the dark at 4° C with the tips in distilled water. As a rule they were not used in experiments for at least 2 days after filling, to allow time for the diffusion of ACh along the narrow portion of the pipettes (length 1·5–2·5 cm).

Multibarrelled micropipettes. Five Pyrex tubes (diameter 6·5 mm; bore 4·5 mm) were fused together side by side to form a symmetrical array (Curtis & Eccles, 1958). They were then drawn out in the device mentioned above, a wider heating coil, more heat and a stronger pull being used, and then filled with glass-distilled water by boiling at atmospheric pressure

* Present address: Department of Physiology, St Mary's Hospital Medical School, London, W. 2.
† Present address: Department of Pharmacology, Dalhousie University, Halifax, N.S., Canada.

for about 30 min. As much distilled water as possible was then sucked out and the appropriate solutions were introduced into the barrels. The tips of the multibarrelled pipettes are initially $< 1\,\mu$ in diameter; to avoid an extremely high electrical resistance, it was necessary to break them so as to have an outside tip diameter of $5–8\,\mu$. The openings of the five barrels at the tip cannot be seen clearly but it is possible to estimate the dimensions of the openings from the electrical resistance of the different barrels (see below). The four outer barrels have very much the same resistance, and presumably similar openings at the tip, but the central barrel has a resistance only one-tenth of that of the others, and therefore a somewhat wider tip opening. After filling, the five-barrelled pipettes were also stored at 4° C.

Assay methods. To reproduce the conditions under which micropipettes are used in physiological experiments, only small quantities of ACh were released, and it was therefore essential to have sensitive methods of assay. Three methods proved comparatively reliable: the assay on the cat's blood pressure, on the dorsal muscle of the leech (both as described by MacIntosh & Perry (1950)), and a micromethod utilizing the dorsal muscle of the leech, described by Szerb (1961). In all experiments the accuracy of the assays was tested by including known solutions of ACh in the sequence of 'unknowns'. Preparations varied much in their sensitivity and reliability. With the best it was possible to perform as many as 30–50 assays. Eleven known solutions tested on two leech muscles gave a coefficient of variation of 16 %. The mean difference between the assay and the true value was only + 0·4 %, showing that there was no systematic deviation. In general, the error of the assays in different experiments expressed as the coefficient of variation came within the range of 16–30 %.

Procedure. After thorough washing with distilled water, the tip of the pipette, held vertically, was immersed in a small volume of 0·15 M-NaCl solution (0·2–4·0 ml., depending on the requirements for assay). Silver leads were inserted in the barrel and in the external solution by which various currents could be passed through the micropipette, their magnitude being read on a series galvanometer, with an accuracy of ± 1 nA. When pulses of current were used, they were monitored by amplifying and displaying on an oscilloscope the voltage drop across a 5 kΩ resistance inserted in series with the pipettes. In several experiments we also examined the release of ACh produced by applying pressure to the contents of a pipette. For this purpose the pipette was connected by a tap to a reservoir containing air at a pressure of 100–150 mm Hg.

Measurements of micropipette resistance. Our standard resistance meter amplified and then rectified the a.c. current flowing through the micropipette during the application of a potential difference of 0·5 V at 50 c/s. Standard resistors were used for calibration.

Some properties of 3·0 M AChCl *solutions.* The viscosity was estimated with an Ostwald-type viscometer held in a water-bath. The temperature of the solution and of the glass-distilled water used for comparison were allowed to equilibrate with that of the water-bath before beginning each run. A series of five runs was done for each fluid; the greatest difference between two readings of the flow time in one series was 2 %. When calculating the coefficient of viscosity, an allowance was made for the somewhat greater density of 3·0 M AChCl. This was estimated by weighing an accurately known volume of solution.

The conductivity of 3·0 M AChCl was measured in a standard conductivity cell at a frequency of 2000 c/s, a conventional bridge with a null-point indicator being used. The cell was immersed in a water-bath and the temperature allowed to reach equilibrium before beginning the measurements.

We also examined the electrophoretic mobility of fine Hysil glass particles (about $10\,\mu$) in 3·0 M AChCl at 25° C. The particles were exposed to known potential gradients in a longitudinal electrophoresis cell, and movements were observed directly with a microscope. The method has been described more fully by Bangham, Pethica & Seaman (1958).

RESULTS

Substantial amounts of ACh may be released from pipettes spontaneously. These amounts are quite variable and they are liable to be so large as to mask any iontophoretic release. It was expected that some ACh would diffuse out of the pipettes, in view of its very high concentration. Moreover, as the pipettes were held vertically it was likely that a certain quantity of ACh solution would flow out because of the hydrostatic head of pressure (usually about 7 cm. of solution).

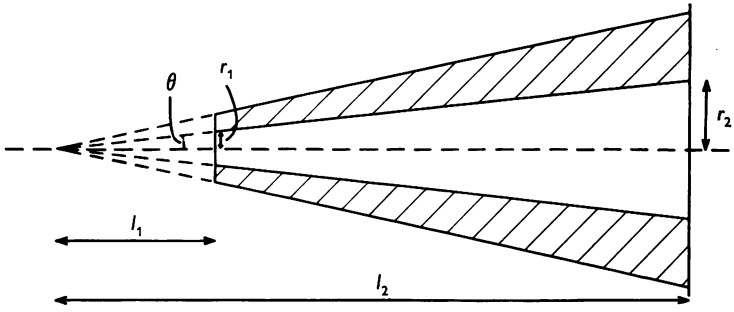

Fig. 1. Theoretical model of the tip of a micropipette seen as a hollow truncated cone.

An estimate of the expected rate of outward diffusion of ACh can be obtained by making several simplifying assumptions: (1) The narrow portion of the micropipette has the shape of a hollow cone, as in Fig. 1. (In fact, the angle of taper is not constant, but the rate of change is quite small in the critical region near the tip (see below).) (2) The concentration (C_i) of ACh in the wider portion of the pipette does not change appreciably in the course of an experiment. (The wide segment contained about 0·2 ml. of solution, i.e. enough ACh to last 10^9 sec at the usual rate of spontaneous outflow.) (3) The diffusion of ACh is considered to take place under steady-state conditions, with a fixed concentration at the wide end of the cone, and zero concentration at the narrow end (several minutes were allowed for equilibrium at the beginning of the experiment). The rate of diffusion is thus constant and should be given by

$$\dot{Q}_D = D\pi r^2 \frac{dc}{dl},$$

where D is the diffusion coefficient of ACh and dc/dl the gradient of concentration inside the pipette. It is evident that the radius r and the length l are mutually related, and that $dl = dr/\tan\theta$, where θ is one-half of the internal angle at the apex of the cone. If dr is thus substituted in the diffusion equation, we have

$$C_i = \int_{r_1}^{r_2} \frac{\dot{Q}_D dr}{D\pi \tan\theta . r^2},$$

from which the diffusion rate ($\dot{Q}_D$) in moles/sec is obtained:

$$\dot{Q}_D = \frac{C_i D\pi \tan\theta}{1/r_1 - 1/r_2}. \qquad (1)$$

Since r_2 is very much wider than r_1 ($r_2 \gg 100r_1$) this equation can be simplified to

$$\dot{Q}_D = C_i D\pi \tan\theta r_1. \qquad (1a)$$

The rate of bulk flow caused by the difference in hydrostatic pressure between the two ends of the pipette should be given by $\dot{V} = (\pi r^4/8\eta)\,(\mathrm{d}p/\mathrm{d}l)$, where η is the coefficient of viscosity of the internal solution and $\mathrm{d}p/\mathrm{d}l$ the gradient of pressure. If $\tan\theta(\mathrm{d}p/\mathrm{d}r)$ is substituted for $\mathrm{d}p/\mathrm{d}l$,

$$p^* = \int_{r_1}^{r_2} \frac{8\eta\dot{V}\,\mathrm{d}r}{\pi\tan\theta r^4}, \quad \text{and therefore} \quad \dot{V} = \frac{3\pi\tan\theta p^*}{8\eta(1/r_1^3 - 1/r_2^3)}, \tag{2}$$

where p^* is the over-all head of pressure. This is again simplified to

$$\dot{V} = \frac{3\pi\tan\theta p^* r_1^3}{8\eta}. \tag{2a}$$

The amount of AChCl coming out by bulk flow (Q_{fl}) is then $\dot{V}C_i$ and the total spontaneous rate of release of ACh ($\dot{Q}$) will be given by

$$\dot{Q} = \dot{Q}_D + \dot{Q}_{fl} = \pi\tan\theta C_i\left(Dr_1 + \frac{3p^* r_1^3}{8\eta}\right). \tag{3}$$

It is clear that the bulk flow, and therefore the total release, will increase very rapidly with the size of the opening at the tip. From equation (3) the ratio of outward movement by bulk flow to that by diffusion is $\dot{Q}_{fl}/\dot{Q}_D = 3p^* r_1^2/8\eta D$. For a typical micropipette the height of the fluid was 7·0 cm; taking into account our estimate of 1·058 for the density of 3·0 M AChCl, this gives a value of 7265 dynes/cm^2 for p^*. Measurements of the viscosity of the solution (η) gave 0·0423 poise, while D can be taken as 10^{-5} cm^2/sec. (In an agar gel, at a concentration of 5×10^{-5} M at 20°C, D is $9{\cdot}8\times10^{-6}$ cm^2/sec (Krnjević & Mitchell, 1960); although diffusion coefficients tend to diminish with increasing concentration, they increase again at really high concentrations, so that the value of D at 3 M may not be very different from that at 10^{-5} M.) Substituting these values, $\dot{Q}_{fl}/\dot{Q}_D = 64{\cdot}4\times10^8 r^2$, or $64{\cdot}4r^2$ if r is given in microns. It follows that outflow should exceed outward diffusion whenever the internal radius at the tip (r_1) exceeds 0·125μ, and outflow should account for over 9/10 of the total release when $r_1 > 0{\cdot}4\mu$ (the corresponding 3 M AChCl micropipette resistance would be in the range of 25–47 MΩ for $r_1 = 0{\cdot}125\mu$, and about 8MΩ for $r_1 = 0{\cdot}4\mu$, as calculated below).

In practice, the internal radius at the tip cannot conveniently be measured with any accuracy, unless the tip is excessively large, but an estimate of r_1 can be obtained from the electrical resistance of the micropipette. If we consider the hollow truncated cone of Fig. 1, the resistance of a segment of length $\mathrm{d}l$ is $\rho_i\,\mathrm{d}l/\pi r^2$, where ρ_i is the specific resistance of the internal solution (here assumed constant). If $\mathrm{d}r/\tan\theta$ is substituted for $\mathrm{d}l$ the total resistance of the barrel is

$$R_i = \int_{r_1}^{r_2} \frac{\rho_i\,\mathrm{d}r}{\pi\tan\theta r^2}, \quad \text{hence} \quad R_i = \frac{\rho_i}{\pi\tan\theta}\left(\frac{1}{r_1} - \frac{1}{r_2}\right). \tag{4}$$

This equation differs at first sight from that derived by Amatniek (1958) who used a slightly differential model. However, since $r_2 \gg r_1$ the equation reduces to

$$R_i = \frac{\rho_i}{\pi\tan\theta r_1}, \tag{4a}$$

which is identical with Amatniek's equation for small values of θ, when $\tan\theta \to \theta$.

When calculating R_i it is usually not justified to assume that ρ_i is a constant. In the region of the tip, ρ_i is likely to be greater than in the bulk of the solution, because the internal concentration is reduced by outward diffusion; yet it is in this region that most of the resistance is found (from equation (4), it is evident that half the total resistance occurs between the tip and the point where $r = 2r_1$). Some idea of the magnitude of any error introduced can be obtained as follows. It is assumed, that, to a first approximation, the internal specific resistance is inversely proportional to the internal concentration of AChCl, i.e. $\rho = K/C$, where K is a constant. For convenience, the outside concentration (C_e) is taken as equal to the external salt concentration (0·15 M). From equation (1), the concentration at any point is

$$C = C_e + (C_i - C_e)\left[\frac{1/r_1 - 1/r}{1/r_1 - 1/r_2}\right].$$

Hence $$\rho = \frac{K(1/r_1 - 1/r_2)}{C_e(1/r_1 - 1/r_2) + (C_i - C_e)(1/r_1 - 1/r)},$$

and $$R_i = \int_{r_1}^{r_2} \frac{K(1/r_1 - 1/r_2)\,dr}{\pi \tan\theta r^2[C_e(1/r_1 - 1/r_2) + (C_i - C_e)(1/r_1 - 1/r)]};$$

the solution of this is

$$R_i = \frac{K(1/r_1 - 1/r_2)}{\pi \tan\theta (C_i - C_e)} \ln \frac{C_i}{C_e}. \tag{5}$$

The equivalent conductance is not independent of concentration, but the error introduced by assuming $\rho = K/C$ is minimized if the corresponding values of ρ are used instead of C, as follows:

$$R_i = \frac{\rho_i}{\pi \tan\theta r_1} \cdot \frac{\rho_e}{\rho_e - \rho_i} \ln \frac{\rho_e}{\rho_i}. \tag{5a}$$

Our previous estimates of R_i should therefore be corrected by a factor approximately equal to $[\rho_e/(\rho_e - \rho_i)] \ln (\rho_e/\rho_i)$.

Measurements of the conductivity of 3·0 M AChCl gave a value of 16·4 Ω cm for ρ_i at 20° C. The resistance of micropipettes (R) was measured with the tips immersed in an external solution of 0·15 M-NaCl, for which ρ_e is 71·2 Ω cm at 20° C, calculated from an equivalent conductance of 93·7 Ω^{-1} cm^2 (obtained by interpolation from the data given in the International Critical Tables). Hence $[\rho_e/(\rho_e - \rho_i)] \ln (\rho_e/\rho_i) = 1{\cdot}30 \ln 4{\cdot}34 = 1{\cdot}91$. Bulk flow of solution is likely to reduce the magnitude of this effect in most pipettes. The actual ratio of the resistance observed with the tip in 0·15 M-NaCl to that in 3 M AChCl was about 1·5 for 2 pipettes with resistances in saline of 8–12 MΩ, 1·15 for a 1 MΩ pipette, and 1·6 for an 80 MΩ pipette. In most calculations the simpler equation was therefore used, except when dealing with very fine tips.

The resistance R_i of the pipette is not necessarily the over-all resistance measured when a current is passed between an electrode in the barrel and one some distance away in an external solution into which the pipette is dipping. There is also an external component of resistance that may or may not be significant. An estimate of the external resistance (R_e) can be made by assuming (cf. Amatniek, 1958) that this resistance approximates to that between two concentric spheres, the inner sphere having a surface area equal to the area of the opening at the tip of the pipette. If the radii of the hypothetical spheres are indicated by r_1^* and r_2^* we now have $dR = \rho_e dr^*/4\pi r^{*2}$, which on integration gives

$$R_e = \frac{\rho_e}{4\pi}\left(\frac{1}{r_1^*} - \frac{1}{r_2^*}\right).$$

Since $r_2^* \gg r_1^*$, this is equivalent to $R_e = \rho_e/4\pi r_1^*$. By definition, $4\pi r_1^{*2} = \pi r_1^2$, since r_1^* is the radius of the imaginary inner sphere, and r_1 the radius of the tip opening. Hence $r_1^* = \frac{1}{2}r_1$ and the equation can be simplified to

$$R_e = \frac{\rho_e}{2\pi r_1}. \tag{6}$$

The over-all resistance is, therefore,

$$R = R_i + R_e = \frac{1}{\pi r_1}\left(\frac{\rho_i}{\tan\theta} + \frac{\rho_e}{2}\right). \tag{7}$$

As $\tan\theta$ is usually $< 0{\cdot}05$, the component of external resistance is in practice unlikely to exceed $1/10R$, unless measurements are made in very dilute external solutions ($< 0{\cdot}1$ M-NaCl). From equation (7) it follows that

$$r_1 = \frac{1}{\pi R}\left[\frac{\rho_i}{\tan\theta} + \frac{\rho_e}{2}\right]. \tag{8}$$

If ρ_i, ρ_e and $\tan\theta$ are known, r_1 can be evaluated in terms of R; one can then predict the magnitude of the spontaneous release of ACh ($\dot{Q}$) for different values of R. To estimate $\tan\theta$, we measured the external taper of the micropipettes between the tip and a point

where the external diameter was about 15 μ. This would include the region between r_1 and $r = 10r_1$, i.e. the length of pipette responsible for 90% of the over-all resistance. For comparison, we also measured the taper between the points corresponding to external diameters (d_e) of 15 and 300 μ, and also between $d_e = 300\,\mu$ and $d_e = 1000\,\mu$. There was relatively little variation between micropipettes. Measurements between the tip and $d_e = 15\,\mu$ on 10 micropipettes gave a mean value of 0·0349 (range 0·025–0·050) for tan θ'. (θ' is one half the *external* angle at the apex of the cone.) The corresponding mean value and range were 0·0249 (0·012–0·029) between $d_e = 15\,\mu$ and $d_e = 300\,\mu$, and 0·0098 (0·067–0·017) between $d_e = 300\,\mu$ and $d_e = 1000\,\mu$. The taper of the lumen (tan θ) was then derived from tan θ' by assuming that the thickness of the glass wall remains a constant fraction ($\frac{1}{2}$) of the outside radius at all points. This could be seen to be approximately true to within a few hundred microns of the tip, as determined by direct examination with a microscope, while previous evidence suggests that the same relationship holds as far as the tip itself (Kitamura, 1958; Fatt, 1961). The internal tangent near the tip, tan θ, was therefore taken as $\frac{1}{2}$ tan θ', that is, a mean of 0·0175, with a probable range of 0·012–0·025.

In a few cases where the tip diameter could be measured, we compared the calculated R with the observed R. For instance, a pipette with an outside tip diameter of 15 μ was assumed to have $r_1 = 3{\cdot}7\,\mu$. For tan $\theta = 0{\cdot}012$ the calculated value of R was 2·36 MΩ, while the actual value was 2·5 MΩ. Another pipette with an outside diameter at the tip of 1·5 μ and tan $\theta = 0{\cdot}033$ gave a calculated R in the range 4·4–8·3 MΩ (because of the relatively fine tip this calculation included the correction $[\rho_e/(\rho_e - \rho_i)] \ln (\rho_e/\rho_i)$); the actual value of R was 7 MΩ. This was quite good agreement; on the other hand, another similar pipette, also with a tip diameter of 1·5 μ but with tan $\theta = 0{\cdot}05$, had a calculated R of 3·2–6·0 MΩ; the observed R was 100 MΩ. This discrepancy was probably due to blocking of the lumen by crystals or particles of dust. Although our solutions were filtered before use, we could not altogether prevent such contamination.

Spontaneous release of ACh

The steady spontaneous release from 14 single-barrelled micropipettes is shown by the points in Fig. 2, the outflow of ACh in moles/sec being plotted on log × log paper against the measured resistance of the corresponding micropipette in MΩ. In a few cases the tips of the pipettes were broken deliberately and the observations repeated with a lower tip resistance. The continuous line in Fig. 2 is the expected spontaneous release calculated from equation (3), values of r_1 being obtained from equation (8) on the assumption that tan θ was 0·0175 for all the pipettes (i.e. $r_1 = 3{\cdot}09/R$; r being in microns and R in megohms). Other values used were $C_i = 3{\cdot}0 \times 10^{-3}$ mole/ml., $D = 1{\cdot}0 \times 10^{-5}$ cm²/sec, and $\eta = 0{\cdot}0423$ poise (our own estimate for 3·0 M-AChCl at 20° C, as already mentioned); p^* was 7265 dynes/cm² for a 7·0 cm fluid column with a density of 1·058. The equation of the line in Fig. 2 was therefore

$$\dot{Q} = 509{\cdot}4\left[\frac{1}{R} + \frac{615}{R^3}\right], \qquad (9)$$

the expected rate of spontaneous release $\dot{Q}$, being in moles × 10^{-15}/sec, and the micropipette resistance in MΩ. The agreement between the line and the experimental points is only rather approximate. It must be

stressed that no attempt was made to fit the points; the various values of ρ, D, η, etc., were either actually measured, or those which seemed the most likely. Tan θ was assumed to be constant, though it was known that it could vary over a range of at least 0·012–0·025. Since tan θ is a squared factor in the final equation, its variations would account for an appreciable

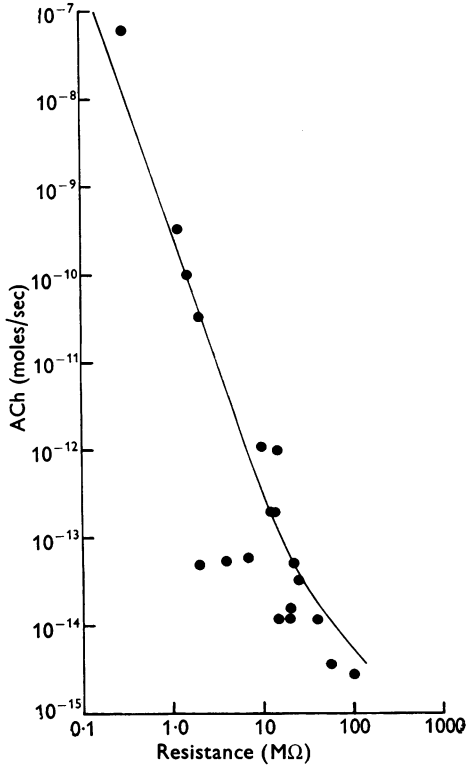

Fig. 2. Spontaneous flux of ACh from micropipettes with different electrical resistances, plotted on log × log paper. Points are experimental observations, while line gives values predicted theoretically (from equation 9) from the tip resistances.

amount of dispersion. However, it is clear that the theoretical line tends to over-estimate the release from tips with a high resistance. It is possible that a lower value of D than 10^{-5} cm^2/sec would be more appropriate, or that tan θ (which is derived from the *external* angle of taper) has been systematically underestimated. In some cases a charged particle blocking the lumen may have been displaced sufficiently by the current flow for the measured resistance to be substantially less than the true resistance. Furthermore, there was usually a positive junctional potential of 10–50 mV between the silver wire in the barrel (in 3 M AChCl) and the silver wire in the external solution (0·15 M-NaCl). This would tend to reduce the outflux

of ACh^+; the effect would be negligible at resistances up to 20 MΩ, but at 50 MΩ this factor could account for a 20 % reduction in $\dot{Q}$, and at 100 MΩ for a 28 % reduction.

The spontaneous release from three multibarrelled pipettes agreed quite well with expectations from equation (9). One pipette had $R = 50$ MΩ and $\dot{Q} = 20 \times 10^{-15}$ mole/sec; another had $R = 25$ MΩ and $\dot{Q} = 50\text{–}100 \times 10^{-15}$ mole/sec; and a third $R = 11$ MΩ and $\dot{Q} = 480 \times 10^{-15}$ mole/sec. This suggests that the effective internal taper, (tan θ) was probably not very different from 0·0175, the mean value assumed in deriving equation (9).

Iontophoretic release of ACh

Estimates of the transport number of ACh were obtained by passing steady currents or pulses through 14 single pipettes, and 2 multi-barrelled pipettes. In each case, the iontophoretic release was determined from the amount of ACh which had been collected, making due allowance for the observed spontaneous liberation. This quantity of ACh (n) was compared with the total electrical charge that had passed through the electrode (Q), and the transport number (t) was calculated from $t = nF/Q$, where F is the Faraday.

Sixty-five values of t were obtained, which gave a mean of 0·421, with a standard error of $\pm 0{\cdot}0418$. The mean values of t in experiments with single and with multibarrelled pipettes were very similar, being 0·431 and 0·410, respectively. The general relation between ACh release and the charge was also derived by calculating the regression of n on Q, all 65 sets of results being used. The regression was highly significant ($f = 9{\cdot}06$, $P < 0{\cdot}01$) and gave the following equation, in which n indicates picomoles of ACh and Q is given in μC: $n = 4{\cdot}59Q + 1{\cdot}06$. This suggests that, *in the aggregate*, the release of ACh was directly proportional to the iontophoretic current (the constant 1·06 has a standard error of $\pm 10{\cdot}7$).

These mean values of t are appreciably higher than might be expected from the estimate of 31 Ω^{-1} cm^2 for the limiting equivalent conductance of ACh^+ at 18° C, given by Fatt (1954). Taking 66 Ω^{-1} cm^2 as the limiting equivalent conductance of Cl^- at the same temperature (from the data given by Conway, 1952), t in a solution approaching infinite dilution would be 0·32, and probably somewhat less at higher concentrations. Whenever possible, individual micropipettes were studied in greater detail to obtain more information about the transport of ACh than was available from the ratios of $n:Q$. In most experiments the limitations of the method of assay precluded a fuller analysis, but in 4 experiments sufficient data were obtained to calculate in each case the regression of the release of ACh on the charge passed through the pipette, and t was obtained after multiplying the regression coefficient by F. The experimental points in the

best series of this kind and the corresponding regression line are shown in Fig. 3. The charge was altered by varying the current intensity between 50 and 150 nA and the duration between 25 and 100 sec. The regression was highly significant ($f = 54{\cdot}3$, $P < 0{\cdot}01$) and the regression coefficient was 2·65 pmole/μC (with a standard error of $\pm 0{\cdot}361$). It will be noticed that the regression line passes very nearly through the origin, the value of y for $x = 0$ being 1·9, with a standard error of 3·2. This is clearly not significantly different from zero, and the regression line is consistent with the postulate that in this experiment the release of ACh was directly

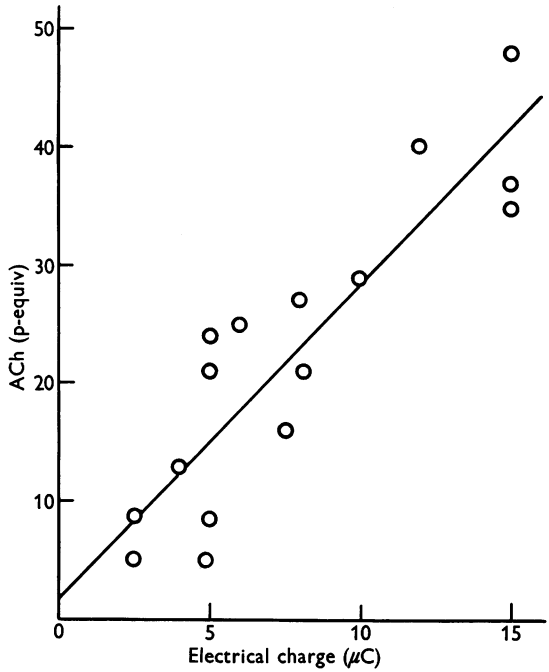

Fig. 3. Open circles show release of ACh by various amounts of electrical charge passed through the barrel containing 3·0 M AChCl of a 5-barrelled micropipette. Calculated regression line gives $y = 2{\cdot}65x + 1{\cdot}9$.

proportional to the iontophoretic current. If the regression line is recalculated so as to go through the origin, the slope is 2·84 instead of 2·65 pmole/μC.

Most of the error in this result was probably due to variations introduced by the method of assay. This can be deduced from 11 assays of known solutions of ACh which were performed 'blind' at intervals during the same experiment. The variance about the regression of these control estimates on the true values came to 18·3, while the variance about the regression of the release of ACh on the charge was 36·9. The difference between these values is not very large, the variance ratio (f) being only 2·0 and the corresponding probability $P > 0{\cdot}1$. This suggests that the transport number during the experiment varied much less

than might appear from the over-all standard error. If we take the figure of 2·65 pmole/μC and calculate the transport number of ACh, t is found to be 0·26 ($\pm$0·035).

It is significant that in these four experiments the estimate of t from the regression coefficient was consistently less than the mean of estimates based upon individual ratios of $n:Q$. The 4 respective pairs of values of t were 0·22 and 0·27, 0·26 and 0·28, 0·27 and 0·42, and 0·35 and 1·03.

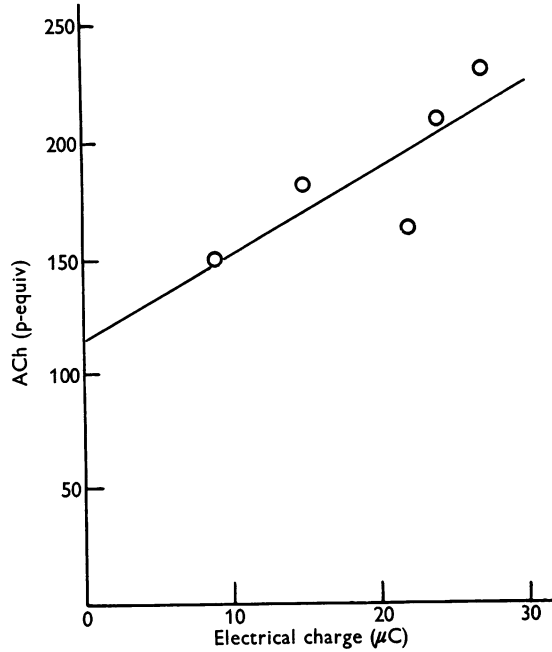

Fig. 4. Open circles show release of ACh by five different amounts of electrical charge passed through one barrel of another 5-barrelled micropipette. Calculated regression line gives $y = 3{\cdot}67x + 114$.

The observations from the last experiment and the corresponding regression line can be seen in Fig. 4, where the current intensity varied between 60 and 150 nA and the duration between 150 and 200 sec. Because of the small number of points, the regression coefficient has only a low degree of accuracy, but the calculated value of t (0·35 S.E. $\pm$ 0·16) is much more consistent with the expected value than the mean of the 5 ratios of $n:Q$ (1·03). The reason for this apparent discrepancy is that the line does not go through the origin: at $x = 0$, $y = 114{\cdot}4$, with a standard error of 34·2. This was probably an extreme case, but various degrees of this kind of behaviour, causing a greater release of ACh than can be accounted for quantitatively by the current flowing through the pipette, may be responsible for the higher values of t calculated from ratios of $n:Q$. Thus the mean of t obtained from the ratios of $n:Q$ for these four pipettes was

0·50, whereas the mean derived from the 4 regression coefficients was 0·28. This last figure, representing the *true* rather than the *effective* transport number, is quite close to the expected value of t, which would be somewhat lower than the value of t at infinite dilution (0·32) on account of the high concentration of AChCl in our pipettes (the transport number of Li in solutions of LiCl is 0·325 at a concentration of 0·01 M, and 0·287 at 1·0 M (Kortüm & Bockris, 1951)). It seems, therefore, that the true transport number of ACh in micropipettes differs little from that observed under ordinary conditions.

In five experiments square pulses of outward current were passed through four single pipettes and one multibarrelled pipette (one barrel contained 3·0 M AChCl, and the other four barrels 2·7 M-NaCl). The pulses were of 4–10 μA, lasting 2–10 msec. They were applied at frequencies of 2–10 c/s, the total number of pulses in one sequence being about 1200. The results of these experiments were included in the 65 estimates used in calculating an over-all mean value of t. Taken by themselves, the 18 ratios of $n : Q$ gave a mean value of t, for pulses, of 0·357 (S.E. $\pm$ 0·0531). This does not differ significantly from the general mean (0·421 $\pm$ S.E. 0·0418). A sufficient number of points for a regression line were obtained in one experiment by varying the amplitude and duration of the pulse: 9 points gave a regression coefficient from which t was calculated to be 0·367 (S.E. $\pm$ 0·057). This value is somewhat higher than was found with steady currents. Part of this difference may have been caused by underestimating the current flow through the pipette, recorded as a voltage pulse across the monitoring resistance. The recorded pulses were not square, showing marked transients at the beginning and the end, which were thought to represent current shorted by the parallel capacitance to earth and were therefore neglected when estimating the total flow of current during the pulses. These transients may have contributed substantially more to the flow of current through the pipette than was believed likely.

When control runs were done by passing pulses of *inward* current of the same magnitude, no extra ACh could be detected in the external solution, showing that, as expected, only outward pulses are effective in releasing ACh^+.

Other controls

Although there was little reason to believe that there would be much interaction between contiguous barrels in multibarrelled pipettes, control tests were performed in two experiments by passing large outward currents through barrels containing 2·7 M-NaCl adjacent to barrels having the usual 3·0 M AChCl. Currents of 1000 nA (compare the usual values of 10–120 nA in physiological experiments), lasting 100 sec, repeatedly failed to cause any detectable release of ACh from the adjoining barrels.

Electro-osmosis

Electrokinetic phenomena usually become negligible at high concentrations of electrolytes, because the thickness of the electrical double layer is much reduced (Freundlich, 1923; Abramson, 1934; Davies & Rideal, 1961). Nevertheless, it seemed of some interest to investigate the possibility that electro-osmosis might contribute substantially to the release of ACh by an applied potential. As it was not practicable to measure electro-osmosis in our pipettes directly, the reverse experiment, which consisted in observing any electrophoretic movement of glass particles in 3·0M AChCl under the influence of an electric field, was performed instead (as described in Methods). The particles were of hard glass (Hysil), comparable with the Pyrex glass used in preparing our micropipettes. Even with the greatest potential gradient available no movement of the glass particles could be detected in 3·0M AChCl; it was, therefore, concluded that the electrophoretic mobility did not exceed $0{\cdot}1\,\mu$ cm/V.sec, the lower limit of resolution of the apparatus.

If we assume that the glass particles are little cylinders, the electrophoretic mobility is identical with the streaming velocity in a capillary containing the same solution, under the same potential gradient (with spheres the electro-osmotic mobility would be 50% greater). The electro-osmotic flow of solution in a capillary of radius r is given by

$$\dot{V} = \pi r^2 u E,$$

where $\dot{V}$ is the volume of flow per second, u the electrophoretic mobility and E the potential gradient. In our conical micropipettes, $E = I(\mathrm{d}R/\mathrm{d}l)$, I being the current flowing through, and R the internal resistance. But $\mathrm{d}R = \rho_i \mathrm{d}l/\pi r^2$; therefore $\dot{V} = I\rho_i u$, and the actual outflux of ACh (in mole/sec) caused by electro-osmosis is $\dot{n}_o = C_i \rho_i I u = Iu/\Lambda_i$, Λ_i being the equivalent conductance of the internal electrolyte. Since the iontophoretic release of ACh is $\dot{n}_i = tI/F$, the ratio of the release by electro-osmosis to the release by iontophoresis is

$$\frac{\dot{n}_o}{\dot{n}_i} = \frac{uF}{\Lambda_i t}. \tag{10}$$

In the present case, $C_i = 3 \times 10^{-3}$ mole/ml., $\rho_i = 16{\cdot}4\ \Omega$ cm, $t = 0{\cdot}42$ and $u \not> 0{\cdot}1\,\mu$.cm/V.sec. Therefore $\dot{n}_o/\dot{n}_i \not> 0{\cdot}113$.

Electro-osmosis would therefore add not more than 11% to the total release (aqueous solutions in contact with glass usually behave as though they were positively charged). This result shows that electro-osmosis is likely to play only a minor role (if any at all) in our experiments. This is, of course, in agreement with the fact that the observed transport number of ACh, calculated on the postulate of iontophoretic movement, agrees reasonably well with expectations.

DISCUSSION

Judging by the results strong solutions of AChCl in micropipettes behave much as they do under macroscopic conditions. On the basis of a simple theoretical model for the tip of the micropipette, it was possible to

predict from the electrical resistance of the pipette the probable rate of spontaneous outflux; the predictions were confirmed at least semi-quantitatively by the experimental observations. Our analysis has emphasized the importance of bulk flow at the tip of pipettes which are held vertically: unless the internal radius at the tip is less than about $0{\cdot}12\,\mu$ (the corresponding tip resistance being about 25–45 MΩ), the outflow is likely to equal or to exceed the outward diffusion. If the tip resistance is appreciably less, the outflow increases very rapidly, at a rate proportional to the cube of the radius. The fact that our observations were made with comparatively long pipettes does not affect appreciably the general validity of these conclusions, because the height of the fluid column is a relatively unimportant factor when compared with the resistance of the tip.

This point is of some practical importance in physiological experiments with micropipettes. It is customary to neutralize the spontaneous output of ACh by applying a suitable constant voltage, tending to draw a 'braking' inward current into the pipette (Del Castillo & Katz, 1955). If one considers only outward diffusion, which is inversely proportional to the tip resistance, one would expect any change of resistance, tending to increase or reduce the output, to be automatically compensated for by a proportional change in the 'braking' current. In fact this will be approximately true only at very high values of the tip resistance where bulk flow is negligible. Moreover, the possibility of neutralizing spontaneous outflow by a braking voltage is limited to a rather small range of tip openings, since the required voltages soon become impracticably large. Thus with a tip resistance of 1 MΩ (corresponding to an internal radius at the tip of about $3\,\mu$), the outflux is 3×10^{-10} mole/sec (see Fig. 2). To balance this by an inward current would require the application of -100 V. Clearly, micropipettes used for the release of drugs by iontophoresis should not have a tip opening with a radius much greater than about $0{\cdot}25\,\mu$, roughly equivalent to an external diameter of about $1\,\mu$. Indeed, one cannot safely record unit activity in the central nervous system with micropipettes containing 3 M-KCl unless the tip is equally small.

Although a large outflow of solution is a possible hazard (if the tip of the pipette should break *in situ*, the tissue may be flooded locally with a powerful pharmacological agent), the bulk flow can be turned to advantage if it is desired to repeat observations on the actions of substances without passing any current through the tissue. From equations ($2a$) and (8), it can be calculated that under a head of pressure of 140 mm Hg, the internal solution should be forced out of the tip at a rate of about 10^{-9} ml./sec. This may seem negligible, but, if the internal concentration is 3 M, this flow is equivalent to the release of ACh at a rate of 3 pmole/sec, or,

in terms of iontophoresis, release by a current of 10^{-6} A (taking $t \doteq 0{\cdot}3$). This is about 10 times the magnitude of current commonly used in physiological experiments.

We have confirmed that such an outflow can actually occur. For instance when a head of pressure of 137 mm Hg was applied to a pipette with a resistance of 22 MΩ, for 300 sec, the extra ACh released was equivalent to 12·9 pmole/sec, corresponding to a flow rate of $4{\cdot}3 \times 10^{-9}$ ml./sec. This method has been used successfully to apply excitatory and inhibitory amino acids to single cortical neurones (Krnjević & Phillis, 1963). In general, however, pressure injection is not a particularly convenient way of applying substances to single cells. The onset and cessation of the release appear to be slower than with iontophoresis, probably owing to a frictional lag in the fine lumen. This can be an important disadvantage if the nerve or muscle cells are rapidly desensitized by the applied agent. The greatest drawback is the difficulty of controlling the rate of flow with any precision. Small changes in the tip resistance may cause large variations in outflow at a given pressure. Thus repeating the above experiment with another micropipette, whose resistance was 60 MΩ instead of 22 MΩ, no release of ACh could be detected over a period of 100 sec. (The total outflow would have been about 1/80 of that observed above, and therefore below the threshold of our assay.) On the other hand when the tip of the first pipette was broken sufficiently to lower the resistance to about 1/4 of the initial value (to 6 MΩ) the output of ACh increased approximately a hundred-fold. Nevertheless, this method can be of value when it is important to eliminate interference by electrical currents, or when one is dealing with substances which are unsuitable for iontophoresis, because they are poorly ionized in solution or not available in a pure form (e.g. in tissue extracts).

Transport number of ACh

The mean effective transport number in 3·0 M AChCl, calculated from all the 65 observed ratios between the release of ACh and the iontophoretic current, was 0·42 (S.E. $\pm$ 0·0418), which is reasonably close to the value expected from the equivalent conductance of ACh^+ at finite dilution (probably somewhat less than 0·32). Under the best conditions, when it was possible to obtain a sufficient number of points to calculate the slope and therefore the 'true' value of t, the agreement was even better: the mean of 4 estimates of t was 0·278 (range 0·22–0·35). The difference between these two means may be due to the occasional anomalous behaviour of pipettes seen when the line relating to the current does not go through the origin (cf. Fig. 4). This cannot be attributed to electro-osmosis, since it should also be simply proportional to the applied voltage. One may perhaps ascribe this phenomenon to rotation of a charged particle

obstructing the lumen, under the influence of the electric field during current flow, which may cause a temporary increase in the spontaneous output of ACh, equivalent to the point where the line in Fig. 4 crosses the Y axis.

It is significant that there was little difference between the transport number observed when short pulses were used instead of a continuous current. It might be thought that the dilution of ACh at the tip of the micropipette would substantially reduce the transport number seen with short pulses or at the beginning of a prolonged current. In fact, if one considers the volume of the tip in the region where AChCl accounts for less than 90 % of the total electrolyte (using equation (6*a*), and assuming that the diluting solution is 0·15 M-NaCl), one can show that the amount of Na^+ inside the tip which might carry an appreciable amount of current is probably $< 10^{-16}$ mole; in terms of charge this is equivalent to $< 10^{-11}$ C, which cannot be expected to play a significant role in current flow at the rate of 10^{-7} A for much longer than 100 *microseconds*.

In view of the fact that a few micropipettes did give an effective transport number for ACh^+ higher than 1·0, one cannot assume that the transport number during applications with any one pipette is necessarily very near 0·42. It is clearly essential to repeat observations with at least one other pipette. Nevertheless, it is also evident that even extreme values of t are unlikely to differ from the mean by a factor of more than 3.

Although electro-osmosis did not play a significant part in our experiments, it is perhaps an important factor under certain conditions. If the internal fluid contains a substance which is either relatively insoluble or little ionized, the zeta potential, and therefore the electro-osmotic mobility of the solution, will be much greater (as long as the ionic concentration is not excessively low). Since the equivalent conductance of a weak electrolyte would be rather small, it is clear from equation 10 that electro-osmosis might then overshadow iontophoresis. This may be an effective way of applying substances in solutions that are unsuitable for iontophoresis.

SUMMARY

1. The release of ACh from glass micropipettes (single and multibarrelled) has been estimated by bioassay.

2. The rate of spontaneous outflux was shown to agree approximately with theoretical predictions over a wide range of tip resistance.

3. When ACh^+ was released by steady iontophoretic currents from micropipettes filled with 3 M AChCl, its mean transport number was 0·42 (n = 65, S.E. $\pm$ 0·0418). A similar value was obtained when short pulses of current were used.

4. This transport number is a little higher than would be expected from conductance measurements in ordinary solutions (about 0·3). Some features of this small discrepancy are discussed.

5. It was shown that electro-osmotic flow is unlikely to play an important role in the release of ACh from these micropipettes, and that a suitable head of pressure may cause an appreciable flow of solution at the tip; this can be used to release ACh without passing a current through the pipette.

We are especially grateful to Dr A. D. Bangham and Mr D. Hardman for their help in connexion with micro-electrophoretic and conductivity measurements, respectively, and to Mr J. G. Rowell of the A.R.C. Statistics Group, Cambridge, for his kind assistance in dealing with certain aspects of the statistical analysis.

REFERENCES

ABRAMSON, H. A. (1934). *Electrokinetic Phenomena and Their Application to Biology and Medicine.* New York: The Chemical Catalog Co.

AMATNIEK, E. (1958). Measurement of bioelectric potentials with micro-electrodes and neutralized input capacity amplifiers. *IRE Transactions on Medical Electronics.* Vol. PGME-10, pp. 3–14.

BANGHAM, A. D., PETHICA, B. A. & SEAMAN, G. V. F. (1953). The charged groups at the interface of some blood cells. *Biochem. J.* **69**, 12–19.

CONWAY, B. E. (1952). *Electrochemical Data.* Amsterdam: Elsevier.

CURTIS, D. R. & ECCLES, R. M. (1958). The excitation of Renshaw cells by pharmacological agents applied electrophoretically. *J. Physiol.* **141**, 435–445.

DAVIES, J. T. & RIDEAL, E. K. (1961). *Interfacial Phenomena.* London: Academic Press.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol.* **128**, 157–181.

FATT, P. (1954). Biophysics of junctional transmission. *Physiol. Rev.* **34**, 674–710.

FATT, P. (1961). Intracellular microelectrodes. *Methods med. Res.* **9**, 381–404.

FREUNDLICH, H. (1923). *Kapillarchemie.* Leipzig: Akademische Verlagsgesellschaft, M.B.H.

KITAMURA, S. (1958). Memoranda on capillary microelectrodes. *Bull. Tokyo med. dent. Univ.* **5**, 387–397.

KORTÜM, G. & BOCKRIS, J. O'M. (1951). *Textbook of Electrochemistry.* Amsterdam: Elsevier.

KRNJEVIĆ, K. & MITCHELL, J. F. (1960). Diffusion of acetylcholine in agar gels and in the isolated rat diaphragm. *J. Physiol.* **153**, 562–572.

KRNJEVIĆ, K. & PHILLIS, J. W. (1963). Iontophoretic studies of neurones in the mammalian cerebral cortex. *J. Physiol.* **165**, 274–304.

MACINTOSH, F. C. & PERRY, W. L. M. (1950). Biological estimation of acetylcholine. *Methods med. Res.* **3**, 78–92.

NASTUK, W. L. (1953). Membrane potential changes at a single endplate produced by transitory application of acetylcholine with an electrically controlled microjet. *Fed. Proc.* **12**, 102.

SZERB, J. C. (1961). The estimation of acetylcholine using leech muscle in a microbath. *J. Physiol.* **158**, 8–9*P*.

WINSBURY, G. J. (1954). Machine for the production of microelectrodes. *Rev. sci. Instrum.* **27**, 514–516.